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# Effects of Alcohol and Oxidative Stress on Liver Pathology: The Role of the Mitochondrion

**Alan Cahill**, **Carol C. Cunningham**, **Masayuki Adachi**, **Hiromasa Ishii**, **Shannon M. Bailey**, **Bernard Fromenty**, and **Adrian Davies**

*From the Department of Pathology, Anatomy and Cell Biology (AC, AD), Thomas Jefferson University, Philadelphia, Pennsylvania; Department of Biochemistry (CCC), Wake Forest University School of Medicine, Winston-Salem, North Carolina; Department of Internal Medicine (MA, HI), School of Medicine, Keio University, Tokyo, Japan; Department of Environmental Health Sciences (SMB), School of Public Health, University of Alabama at Birmingham, Birmingham, Alabama; and INSERM U-481 (BF), Hôpital Beaujon, Clichy Cedex, France.*

## Abstract

This article represents the proceedings of a symposium at the 2001 Research Society on Alcoholism meeting in Montreal, Canada. The chairs were Alan Cahill and Carol C. Cunningham. The presentations were (1) Mitochondrial regulation of ethanol-induced hepatocyte apoptosis: possible involvement of pro-apoptotic Bcl-2 family protein Bax, by Masayuki Adachi and Hiromasa Ishii; (2) Effects of ethanol on mitochondrial reactive oxygen species production and oxidative protein modification, by Shannon M. Bailey; (3) Acute ethanol binges elicit widespread oxidative mitochondrial DNA damage and depletion: protective effects of antioxidants and inhibitors of ethanol metabolism, by Bernard Fromenty; and (4) Effects of chronic ethanol consumption upon hepatic mtDNA oxidative modification and depletion, by Alan Cahill and Adrian Davies.

### Keywords

Mitochondria; Ethanol; Oxidative Stress; Mitochondrial DNA; Apoptosis

In chronic ethanol consumers, one of the earliest alterations in the liver that adversely affects normal hepatic function occurs in the mitochondrion. Changes include ultrastructural alterations (Kiessling et al., 1964; Porta et al., 1965) and depressions in activities associated with the oxidative phosphorylation system (Cunningham et al., 1990; Hoek, 1994). These changes are potentially very important, given the central role of the mitochondrion in maintaining the hepatic energy state, i.e., normal concentrations of adenosine triphosphate (ATP). Recently, evidence has accumulated that indicates that ethanol-elicited depressions in normal mitochondrial function may be mediated, at least in part, by increased oxidative damage to mitochondrial proteins, lipids, and DNA. This article includes a presentation by Dr. Bailey on the effects of acute and chronic ethanol consumption in increasing the production of mitochondrial reactive oxygen species (ROS) in the liver. Data are presented that illustrate that ethanol metabolism stimulates ROS production at the flavin mononucleotide (FMN) in the nicotinamide adenine dinucleotide (reduced form; NADH) dehydrogenase complex and at ubiquinone in the cytochrome $bc_1$ complex. Also presented in this article is evidence that increased ROS production, combined with decreases in activities associated with the

Reprint requests: Dr. Alan Cahill, Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, 1020 Locust St., Philadelphia, PA 19107; Fax: 215-923-2218; E-mail: E-mail: alan.cahill@mail.tju.edu.

mitochondrial glutathione antioxidant system, correlates with oxidative damage to both mitochondrial proteins and mitochondrial DNA (mtDNA).

The summaries of Drs. Fromenty and Cahill emphasize the damage that occurs to the mitochondrial genome, which encodes for 13 polypeptides, all of which are essential components of the oxidative phosphorylation system. These studies illustrate that mtDNA can be damaged both by acute and chronic ethanol exposure and that this damage is demonstrable in human alcoholics and rodent animal models. The interplay between ethanol consumption and the age of the ethanol consumer on the degree of oxidative damage to DNA is also presented. Previous studies by Dr. Ishii's group provided evidence that hepatocytes exposed to acute doses of ethanol undergo apoptosis, which is linked to oxidative stress (Kurose et al., 1997). In this article Dr. Adachi provides more details on the mechanism of ethanol-stimulated apoptosis, including an outline of the evidence for an interaction between Bax and the voltage-dependent anion channel (VDAC), the latter a component of the permeability transition pore (PTP). The presentations are summarized below.

## MITOCHONDRIAL REGULATION OF ETHANOL-INDUCED HEPATOCYTE APOPTOSIS: POSSIBLE INVOLVEMENT OF PROAPOPTOTIC BCL-2 FAMILY PROTEIN BAX

**Masayuki Adachi and Hiromasa Ishii**

Apoptosis is recognized to be present in both clinical and experimental alcoholic liver disease. Although the mechanism of hepatocellular apoptosis in chronic or acute alcoholic liver disease is proposed to be regulated by various factors, such as oxidative stress and inflammatory cytokines (Ishii et al., 1997; Nanji, 1998), little is known about the intracellular mechanisms by which ethanol intoxication induces the apoptotic machinery in hepatocytes. Our previous study indicates that short-term ethanol intoxication induces oxidative stress followed by mitochondrial dysfunction and apoptosis in cultured rat hepatocytes (Kurose et al., 1997). Recent evidence has demonstrated that mitochondria play a critical role in apoptosis by releasing apoptosis-inducing factors, such as cytochrome *c*, from mitochondria into cytosol. In many systems, apoptosis is associated with a loss of mitochondrial membrane potential, which may correspond to an increase in mitochondrial membrane permeability, referred to as the mitochondrial permeability transition (MPT).

This study was designed to clarify the precise mechanism of ethanol-induced hepatocellular apoptosis and especially to evaluate the role of the Bcl-2–family protein Bax. Short-term treatment with ethanol (50 mmol/liter) induced oxidative stress within mitochondria, MPT, cytochrome *c* release, and apoptosis. Cyclosporin A, which inhibits formation of the PTP, effectively attenuates MPT, cytochrome *c* release, and apoptosis. These observations indicate that MPT is essential for ethanol-induced hepatocellular apoptosis (Higuchi et al., 2001). Moreover, neither activation of caspase-8 nor cleavage of Bid is observed in ethanol-induced hepatocellular apoptosis. Because acute ethanol triggers a Bid/caspase-8–independent mechanism, it may induce apoptosis via a different mechanism from Fas/tumor necrosis factor-α–induced apoptosis in hepatocytes.

Although we have emphasized the role of MPT in ethanol-induced hepatocellular apoptosis, evidence indicates that 15-kDa cytochrome *c* is too large to pass through the PTP, which allows only molecules smaller than 1.5 kDa to pass though. Thus, the precise mechanism by which cytochrome *c* leaves mitochondria during apoptosis is a major unanswered question. The role of the proapoptotic Bcl-2–family protein Bax in the induction of cytochrome *c* release and apoptosis was evaluated when hepatocytes were exposed to ethanol. It was recently reported that Bax is translocated from the cytosol to the mitochondria at an early stage of apoptosis and

triggers cytochrome *c* release from the mitochondria (Jurgensmeier et al., 1998; Wolter et al., 1997). Recent observations have given rise to proposals by which Bax affects the mitochondria during apoptosis, and one potential mechanism is that Bax proteins oligomerize to form a pore channel in the mitochondrial outer membrane (Antonsson et al., 2000). In another potential mechanism, it was recently suggested that Bax interacts with the PTP to induce cytochrome *c* release (Shimizu et al., 1999). We found that the proapoptotic Bcl-2–family protein Bax was translocated from the cytosol to the mitochondria when cultured hepatocytes were exposed to 50 mmol/liter of ethanol. Oligomerization of Bax protein was not observed by the treatment with ethanol. However, Bax formed a complex with the VDAC, a component of PTP. In another set of experiments, microinjection of anti-VDAC antibody into the cytosol of hepatocytes effectively attenuated the ethanol-induced hepatocellular apoptosis, suggesting that the opening of the VDAC channel is essential for the induction of apoptosis.

In conclusion, cytochrome *c* release from mitochondria via PTP was the key step in the execution of ethanol-induced hepatocellular apoptosis. During acute ethanol-induced hepatocellular apoptosis, Bax, which is translocated to the mitochondrial membrane and forms a Bax/VDAC complex, may trigger conformational changes in the PTP, making it large enough to allow passage of cytochrome *c* from the mitochondria.

## EFFECTS OF ETHANOL ON MITOCHONDRIAL REACTIVE OXYGEN SPECIES PRODUCTION AND OXIDATIVE PROTEIN MODIFICATION

**Shannon M. Bailey**

Recent findings suggest that the mitochondrion may be a significant source of ROS in hepatocytes when exposed to ethanol acutely or chronically. Observations indicate that the ability of ethanol to increase mitochondrial ROS production is associated with its metabolism, with ethanol-related alterations to the mitochondrial oxidative phosphorylation system, or both. Moreover, chronic ethanol exposure has been shown to have a detrimental effect on cellular antioxidant defense systems, specifically glutathione peroxidase-1. Furthermore, oxidative modification and inactivation of mitochondrial proteins may be one mechanism by which ethanol disrupts the structure and function of mitochondria and contributes to the pathology observed in the livers of chronic alcohol abusers.

It is well established that under normal physiologic conditions, the mitochondrial electron transport chain is the major source of ROS within cells, due to the leakage of unpaired electrons to molecular oxygen as they are being transported down the respiratory complexes (Boveris and Cadenas, 1997; Boveris and Chance, 1973). There are two main sites in the respiratory chain where ROS generation is proposed to occur: the NADH dehydrogenase complex (complex I) and the ubiquinone/cytochrome *c* reductase complex (complex III). What makes these two sites candidates for ROS production is that the FMN coenzyme present in complex I and the ubiquinone of complex III can both exist in a semiquinone anion form that contains an unpaired electron. It is this unpaired electron that can be donated to molecular oxygen to form the superoxide anion. We propose that the ability of ethanol to further stimulate mitochondrial ROS at these sites is directly linked to its metabolism.

When an individual consumes moderate amounts of ethanol, most of it will be metabolized by the enzyme alcohol dehydrogenase in the liver; this metabolism results in the generation of the reactive metabolite acetaldehyde and NADH. Acetaldehyde is further oxidized by hepatic aldehyde dehydrogenase to acetate, which also results in the production of NADH. As a result of the oxidation of ethanol and acetaldehyde, there is a significant increase in cytosolic and mitochondrial NADH concentrations, respectively, and this increases the availability of oxidizable NADH to the mitochondrion. Thus, during ethanol oxidation, mitochondrial ROS

production would be stimulated due to the increased flux of reducing equivalents into the electron transport chain.

Studies by our group (Bailey and Cunningham, 1998; Bailey et al., 1999) have demonstrated that the mitochondrion is a significant source of ROS when hepatocytes are incubated with ethanol. When hepatocytes from ethanol-naïve rats were incubated with ethanol (1 and 10 mM), the production of ROS was related to the concentration of ethanol. Hepatocyte viability was decreased slightly, but significantly, after incubation with these low concentrations of ethanol, which suggests a connection between oxidant levels and toxicity. The metabolism of ethanol was required for the increase in ROS because pretreatment with 4-methylpyrazole, an inhibitor of alcohol dehydrogenase, attenuated the ethanol induction of ROS levels and protected against the ethanol-related decrease in viability. Likewise, the aldehyde dehydrogenase inhibitor cyanamide also suppressed the ethanol-elicited increase in ROS by inhibiting NADH production via the oxidation of acetaldehyde in the mitochondrion. These observations emphasize that ROS production in the presence of acute doses of ethanol was related to the generation of NADH.

To provide additional evidence for a role of mitochondria in acute ethanol-related increases in ROS production, hepatocytes from ethanol-naïve rats were pretreated with mitochondrial respiratory chain inhibitors. Antimycin inhibits electron transfer from cytochrome $b_H$ to ubiquinone during the Q cycle of complex III. Thus, antimycin has two effects within cells. First, it inhibits ATP synthesis by preventing electron flow through complex III. As a consequence of this blockage, its second effect is to increase the concentration of the ubiquinone semiquinone anion in the Q cycle, which would facilitate the transfer of its unpaired electron to molecular oxygen to form superoxide (Turrens et al., 1985). Thus, in the presence of antimycin, mitochondrial ROS generation would be increased due to the continuous oxidation of endogenous substrates and would be further stimulated by the additional reducing equivalents (i.e., NADH) generated during ethanol and acetaldehyde metabolism.

Including antimycin in the hepatocyte incubations increased the production of ROS dramatically—approximately 10-fold—and, as predicted, coincubation with ethanol further increased ROS levels (Bailey and Cunningham, 1998; Bailey et al., 1999). As expected, pretreatment with 4-methylpyrazole and cyanamide attenuated the ethanol-elicited increase in ROS production. Thus, these observations are consistent with the ubiquinone semiquinone anion associated with mitochondrial complex III contributing to the production of ROS during ethanol oxidation. The excess reducing equivalents (i.e., NADH) generated during ethanol oxidation are shuttled into mitochondria. As a consequence of this increased flux of electrons into mitochondria, the electrons carriers in complex III will be in a more reduced form, which will facilitate the transfer of the unpaired electron of the ubiquinone semiquinone anion to molecular oxygen to generate superoxide.

An additional site for the production of ROS during acute ethanol exposure is the NADH dehydrogenase complex, in which superoxide anions are produced during the reoxidation of the FMN coenzyme. As mentioned previously, the FMN also exists in a semiquinone anion form, and when in this form it can transfer its unpaired electron to molecular oxygen to generate superoxide. To investigate the involvement of the FMN semiquinone anion in ethanol-induced ROS formation, two mitochondrial inhibitors were used that inhibit electron transfer within complex I, but at different sites. Rotenone blocks the transfer of electrons from the iron-sulfur center N-2 to ubiquinone, which prevents the electron flow from NADH-linked substrates into complex III; however, reduction of molecular oxygen to the superoxide anion can still occur at the FMN site of complex I. In contrast, diphenyliodonium (DPI) irreversibly binds with flavin-containing enzymes, and, as a consequence, it will inhibit electron flow through the FMN and diminish superoxide production in both complexes I and III. Incubation with rotenone

significantly increased ROS production, which would be expected because FMN can still accept electrons; ROS production was further stimulated by ethanol, whereas pretreatment with DPI significantly attenuated the ROS production in hepatocytes from untreated and ethanol-treated cells (Bailey et al., 1999). A comparison of the actions of DPI and rotenone indicates that if FMN continues to be an electron acceptor, ROS production can be stimulated by ethanol at this site in the respiratory chain. These data establish that during ethanol oxidation, mitochondrial production of ROS is stimulated both at the FMN site in complex I and during the ubiquinone Q cycle of complex III.

Chronic consumption of ethanol significantly increases the steady-state levels of ROS in hepatocytes isolated from animals fed the Lieber-DeCarli ethanol liquid diets (Bailey and Cunningham, 1998, 1999; Bailey et al., 1999; Ivester et al., 1995). This effect of chronic ethanol was observed in rats maintained on either a high-fat ethanol-containing diet (35% fat) or a diet that contained only 12% of total calories as fat. Moreover, hepatocytes from ethanol-fed rats also have significantly lower levels of ATP after incubation periods (Bailey and Cunningham, 1999; Ivester et al., 1995); this is due to chronic ethanol–related defects in the components of the oxidative phosphorylation system (Cunningham et al., 1990; Hoek, 1994). Thus, hepatocytes isolated from rats fed ethanol chronically are significantly less viable during incubation as compared with controls; this is related to both the increase in ROS production and the inability to maintain ATP levels.

It is highly probable that alterations to the mitochondrial electron transport chain contribute to the increase in ROS production observed in hepatocytes isolated from ethanol-fed animals. It is well established that chronic ethanol consumption induces lesions in complexes I and III of the mitochondrial electron transport chain and that these alterations might result in an increased transfer of electrons to molecular oxygen, thus stimulating superoxide and hydrogen peroxide levels. First, Thayer et al. (1980) demonstrated that the levels of the iron-sulfur centers in complex I were decreased as a result of chronic ethanol consumption. This would have the effect of impeding electron transfer from FMN, which would result in the accumulation of higher concentrations of the semiquinone anion form of FMN and enhance superoxide production. Furthermore, cytochrome *b* levels in complex III are decreased as a result of chronic ethanol feeding (Coleman and Cunningham, 1990; Thayer and Rubin, 1981). This defect would decrease the rate of reoxidation of the ubiquinone semiquinone anion within the Q cycle of complex III, thus increasing its steady-state levels in the ethanol-fed hepatocyte, which would also be expected to increase generation of ROS. Moreover, studies by García-Ruiz et al. (1997) have suggested that the ethanol-related increase in tumor necrosis factor-α release might mediate an increase in ROS at the ubiquinone site of complex III through alterations in lipid-signaling pathways. These observations suggest strongly that the increase in ROS production observed in hepatocytes from rats fed ethanol can be attributed at least in part to ethanol-related lesions in the mitochondrial oxidative phosphorylation system.

When investigating those mechanisms that lead to increased ROS, lipid peroxides, and free radicals in livers exposed to ethanol, it is also important to determine the effect of chronic ethanol exposure on those antioxidant defense mechanisms that function to protect hepatocytes and their subcellular organelles from oxidative damage. A recent study (Bailey et al., 2001) suggests that the chronic ethanol–related increase in hepatocyte ROS levels may be linked to depressed activity of the hydrogen peroxide scavenger glutathione peroxidase-1. Chronic ethanol feeding significantly decreased the activity of glutathione peroxidase-1 by 30 and 40% in mitochondria and cytosol, respectively, as compared with controls. The data that demonstrate a decrease in glutathione peroxidase-1 activity and an increase in ROS production after chronic ethanol consumption, when taken together, provide strong evidence to support the hypothesis that chronic ethanol exposure creates an oxidative and potentially damaging environment within the hepatocyte and specifically within the mitochondria.

It is known that proteins are readily oxidized by various ROS (Berlett and Stadtman, 1997) by the direct oxidative attack of specific amino acid residues and that this results in the generation of carbonyl groups. Indeed, chronic ethanol consumption significantly increased the concentration of protein carbonyls in liver, with a 25% increase in cytosolic and a 60% increase in mitochondrial protein carbonyls as a result of chronic ethanol feeding (Bailey et al., 2001). These data provide strong evidence that chronic ethanol consumption induces oxidative damage to an important component of the cellular machinery—proteins—and, more important, they demonstrate that mitochondrial proteins are more vulnerable to oxidative stress after chronic ethanol consumption. Therefore, it is likely that ethanol-related increases in ROS might have the effect of inactivating mitochondrial proteins, and this would diminish mitochondrial function and ultimately lead to the dysfunction of mitochondria in the chronic alcohol abuser.

## ACUTE ETHANOL BINGES ELICIT WIDESPREAD OXIDATIVE MITOCHONDRIAL DNA DAMAGE AND DEPLETION: PROTECTIVE EFFECTS OF ANTIOXIDANTS AND INHIBITORS OF ETHANOL METABOLISM

### Bernard Fromenty

Because ethanol intoxication increases hepatic ROS formation and mtDNA is highly sensitive to oxidative stress, ethanol-induced mtDNA damage could be expected. We recently assessed the effect of a single dose of ethanol (5 g/kg, orally) on hepatic DNA in mice. Whereas nuclear DNA integrity was not altered, mtDNA was extensively damaged (Mansouri et al., 1999). Two hours after the binge, supercoiled mtDNA had disappeared, with a concomitant increase in the proportion of linear (full-length) mtDNA, together with shorter mtDNA fragments. These results indicated that many mtDNA molecules had undergone linearization and fragmentation, probably due to mtDNA strand breaks.

Extensively damaged mtDNA molecules are probably degraded by mitochondrial nucleases (Croteau et al., 1999). Two hours after ethanol administration, mtDNA was depleted, suggesting complete degradation of numerous mtDNA copies (Mansouri et al., 1999). This mtDNA depletion was prevented by 4-methylpyrazole, an inhibitor of both alcohol dehydrogenase and cytochrome P-450 2E1, indicating that mtDNA degradation was induced by ethanol metabolism and not by ethanol itself. Melatonin, an antioxidant, also afforded protection, suggesting that mtDNA degradation was triggered by alcohol-induced radical formation (Mansouri et al., 1999). In a more recent study, we found that acute ethanol administration also depletes mtDNA in the brain, heart, and skeletal muscles, unless 4-methylpyrazole or melatonin is coadministered (Mansouri et al., 2001).

The mtDNA depletion caused by acute ethanol intoxication is, however, transient (Mansouri et al., 1999). Indeed, normal levels of hepatic mtDNA are restored 4 hr after the alcoholic binge, and after 24 hr, higher than normal concentrations of mtDNA are observed. The in vivo incorporation of [$^3$H] thymidine into hepatic mtDNA is markedly increased from 2 to 10 hr after ethanol administration (Mansouri et al., 1999); this suggests that accelerated mtDNA replication (perhaps associated with mtDNA repair) mediates rapid mtDNA recovery. It is interesting to note that the overshoot of mtDNA levels and synthesis is also observed after mtDNA depletion in brain, heart, and skeletal muscles (Mansouri et al., 2001). Levels of mtDNA are known to be tightly controlled in mammalian cells (Tang et al., 2000), and several regulatory mechanisms are swiftly set in motion to regain or maintain normal mtDNA pools (Holt et al., 2000).

In contrast to the transient effect of a single dose of ethanol, repeated doses seem to have more durable effects on mtDNA levels. Indeed, daily ethanol administration for 4 days induces hepatic mtDNA depletion that persists for 48 hr after the last ethanol dose in mice (Demeilliers

et al., unpublished data, 2002). Moreover, the overshoot of mtDNA levels is not detected later on. It is interesting that mtDNA replication is impaired durably after the fourth ethanol administration, but DNA polymerase γ activity is unimpaired, and messenger RNA levels of the mitochondrial single-strand DNA-binding protein are significantly increased in the livers of the intoxicated mice. Because the mitochondrial single-strand DNA-binding protein is up-regulated whenever the mtDNA copy number needs to be enhanced (Schultz et al., 1998), this result suggests that repeatedly intoxicated mice have an impaired mitochondrial ability to resynthesize mtDNA, despite an apparently adequate up-regulation of nuclear-encoded, mtDNA replication–enhancing factors. In keeping with this view, long polymerase chain reaction experiments indicate that hepatic mtDNA harbors strand breaks, abasic sites, or both and possibly other DNA lesions able to block the DNA polymerase. Finally, hepatic thiobarbituric acid-reactive substances and the cytochrome P-450 2E1 protein are durably increased after the last ethanol intoxication, but not after a single binge, thus suggesting that oxidative stress and lipid peroxidation play a significant role in the prolongation of mtDNA depletion after repeated ethanol intoxication. All together, these results suggest that after repeated ethanol doses, the accumulation of unrepaired mtDNA lesions blocks the progress of polymerase γ on mtDNA and prevents adaptive mtDNA resynthesis, causing prolonged hepatic mtDNA depletion.

Even before these investigations in mice (Mansouri et al., 1999, 2001), we had hypothesized that alcohol intoxication could increase the incidence of hepatic mtDNA mutations in humans. To test this hypothesis, we prepared hepatic DNA from 50 alcoholic patients with different hepatic lesions and 62 age-matched nonalcoholic controls. We first looked for the presence of a 4977-base pair (bp) mtDNA deletion (Fromenty et al., 1995), referred to as the *common deletion* because it is found in very old individuals or patients with mitochondrial cytopathies (Fromenty and Pessayre, 1995). We then searched for other large mtDNA deletions in these patients (Mansouri et al., 1997). Overall, single or multiple hepatic mtDNA deletions were found in 24% of alcoholic patients, whereas only 3% of controls had one mtDNA deletion (Fromenty et al., 1995; Mansouri et al., 1997). A higher prevalence of hepatic mtDNA deletions in patients with alcoholic liver disease than in those with nonalcoholic steatohepatitis was also observed by another group (Caldwell et al., 1999), and alcohol intoxication was found to cause a transient short mtDNA deletion, which disappeared when alcohol was stopped (Tsuchishima et al., 2000), in white blood cells. Thus, numerous mtDNA deletions may occur in the tissues of alcoholic patients.

The large hepatic mtDNA deletions that were observed in alcoholic patients were all flanked by long or short tandem repeats (Mansouri et al., 1997). Such deletions between repeats may occur during mtDNA replication due to slipped mispairing of homologous sequences separated by several thousand base pairs (Berneburg et al., 1999; Fromenty and Pessayre, 1995). It is interesting that slipped mispairing between remote DNA sequences could be favored by DNA strand breaks or any other events able to hamper the progression of the DNA polymerase on its template (Berneburg et al., 1999; Zhang et al., 2000). Thus, the mtDNA strand breaks and abasic sites that we detected in our murine model (Mansouri et al., 1999, 2001) are a probable explanation for the appearance of hepatic mtDNA deletions in alcoholic patients (Fromenty et al., 1995; Mansouri et al., 1997).

The exact role of mtDNA depletion and mutations in the physiopathology of alcoholic liver disease is currently unknown. However, it is noteworthy that in our murine model of repeated ethanol intoxication (Demeilliers et al., unpublished data, 2002), mtDNA depletion was associated with the presence of mitochondrial ultrastructural abnormalities and microvesicular steatosis in some hepatocytes. Moreover, in our alcoholic patients, mtDNA deletions were found almost exclusively in patients with microvesicular steatosis (Fromenty et al., 1995; Mansouri et al., 1997). Because this lesion is the consequence of severe mitochondrial

dysfunction in the liver (Fromenty and Pessayre, 1995), oxidative damage to mtDNA and other key mitochondrial components (i.e., proteins of the oxidative phosphorylation system, cardiolipin) may constitute a primary event during ethanol-induced microvesicular steatosis.

## EFFECTS OF CHRONIC ETHANOL CONSUMPTION UPON HEPATIC MTDNA OXIDATIVE MODIFICATION AND DEPLETION

**Alan Cahill and Adrian Davies**

Mitochondrial DNA is double stranded and circular and represents less than 1% of total cellular DNA. In the rat it is 16,300 bp long (16,569 bp in humans) and encodes for 22 transfer RNA molecules, 2 ribosomal RNA molecules (components of the mitochondrial ribosomes), and 13 polypeptides that make up specific components of the electron transport chain (Gadaleta et al., 1989). In the liver, chronic ethanol consumption is known to increase both intra- and extramitochondrial ROS production (Bailey and Cunningham, 1998; Ishii et al., 1996). Mitochondrial DNA is especially susceptible to attack by these ROS because of its lack of protective histones and because of its location within the mitochondrion, i.e., in close proximity to the electron transport chain (Nass, 1969), a significant locus for free-radical production (Boveris and Chance, 1973). In this symposium, data were presented showing how both acute and chronic ethanol feeding can result in a decrease in rat hepatic mtDNA content. In the chronic model, mtDNA depletion coincided with increased oxidative modification of the DNA, suggesting a role for ROS. Aging enhanced the effects of chronic ethanol feeding on mtDNA content. In addition, data were presented that strongly question the role of endonuclease G in mtDNA homeostasis.

Previous data from Mansouri et al. (2001) have shown that acute ethanol dosing to mice results in depletion of hepatic mtDNA, with a concomitant conversion of supercoiled DNA to a linearized conformation. Depletion of mtDNA was maximal after 2 hr, decreasing to 46% of control values. We investigated the effects of a single acute dose of ethanol (5 g/kg by gavage) to male rats over 12 hr. Mitochondrial DNA was depleted gradually over the course of 8 hr, decreasing to 60% of its initial value. After this, however, mtDNA content began to increase rapidly and after 12 hr reached a value approximately 140% of its initial content. The mtDNA depletion seen during the first 4 hr after ethanol administration was paralleled by a conversion of supercoiled mtDNA to the open circular conformation, indicative of a free radical–based mechanism of DNA damage. We conclude from these studies that a single acute dose of ethanol to male rats causes a decrease in hepatic mtDNA and results in compensatory mechanisms that rapidly restore its content to a level significantly in excess of its pretreatment value. In these respects, the rat seems to behave similarly to the mouse (Mansouri et al., 1999), albeit in a significantly less sensitive manner. As a comparison to the acute studies, we examined the effect of chronic ethanol feeding on hepatic mtDNA content. Male rats were maintained on the Lieber-DeCarli diet for varying periods of time up to 1 year. Mitochondrial DNA levels were found to decrease significantly when compared with those of pair-fed controls (40% decrease after 4–6 months; 50–60% decrease after 1 year). This depletion was found to coincide with significant increases in oxidative modification of the mtDNA, as assessed by 8-hydroxydeoxyguanosine levels (40% increase after 4–6 months; 130% increase at 1 year, relative to paired controls).

Mitochondrial endonuclease G is a nuclear-encoded enzyme that is imported into the mitochondrion. It exists as a homodimer of 25- to 29-kDa subunits and preferentially cleaves DNA at polydeoxyguanosine residues, with its activity having dual pH optima at pH values of 5.5 and 7.5. It has been reported to be involved in mtDNA homeostasis, although its precise role has remained somewhat enigmatic. It has alternatively been suggested to be involved in mtDNA repair (Gerschenson et al., 1994; Houmiel et al., 1991), replication (Cote and Ruiz-

Carrillo, 1993), and degradation (Ikeda and Ozaki, 1997). We examined its role in mtDNA homeostasis and the effects of chronic ethanol feeding on its activity. Endonuclease G was partially purified from control and alcoholic livers (rats fed ethanol for 1 year). It was then incubated with mtDNA isolated from the same livers. Mitochondrial DNA isolated from ethanol-fed animals was found to be significantly more resistant to endonuclease G–mediated digestion, irrespective of whether the enzyme had been isolated from the livers of control or ethanol-fed animals. Further investigation revealed that oxidative modification of mtDNA, as incurred during chronic ethanol feeding (Cahill et al., 1999), inhibited endonuclease G activity. This led us to the initial conclusion that ethanol-mediated oxidative damage of mtDNA impeded the activity of endonuclease G and thus interfered in its potential involvement in mtDNA homeostasis. To more accurately investigate the role of this enzyme in controlling mtDNA content, we performed detailed localization studies on fractionated mitochondria. Results indicated that endonuclease G is actually located on the outside of the inner mitochondrial membrane—more accurately, within the intermembrane space—and can be released from the mitochondria along with cytochrome *c* during formation of a permeability transition. Because mtDNA is located internal to the inner mitochondrial membrane, we conclude that endonuclease G has no role to play in ethanol-elicited mtDNA depletion. Moreover, its location suggests that it is not involved in mtDNA homeostasis.

The two major observations concerning the effects of ethanol on hepatic mtDNA—i.e., increased oxidative damage and decreased content—can also be seen during the aging process. Hepatic mtDNA copy number in 27-month-old rats is decreased by 50% when compared with that of 6-month-old animals (Barazzoni et al., 2000). Additionally, levels of 8-oxodeoxyguanosine are increased 3-fold in hepatic mtDNA from 24-month-old rats when compared with those of 4-month-old animals (Ames et al., 1995). The molecular mechanisms underlying these two phenomena, as well as any relationship between them, have yet to be elucidated. We investigated the effects of aging on hepatic mtDNA depletion in 2-month-old, 12-month-old, and 24- month-old animals that had been fed a short-term (21-day) chronic ethanol–containing diet before death and compared these effects with those from animals of the same age that had been pair-fed a control liquid diet. Aging was found to have little effect on the mtDNA content in 12-month-old control animals (15% depletion when compared with 2-month-old animals). After 24 months, however, mtDNA levels had decreased by a further 45%. These results are similar to those reported by Ames et al. (1995) for chow-fed animals and suggest an age-related acceleration in mtDNA depletion during the second year of the rats' life span. This may be due to accelerated mtDNA degradation or decreased mtDNA replication or repair. Short-term chronic ethanol feeding before death was found to cause a significant age-related depletion in hepatic mtDNA levels. Whereas 2-month-old animals possessed mtDNA levels similar to those of paired controls, shortterm ethanol feeding of 12-month-old and 24-month-old animals resulted in mtDNA depletions of 70% and 90%, respectively, when compared with those of 2-month-old controls. Moreover, these depletions were accompanied by widespread mtDNA degradation. The decrease seen in the 12-month-old animals was greater than that seen with the long-term (12-month) chronic feeding model in 2-month-old animals; this may reflect an adaptive response to ethanol consumption in the young animals. Statistical analyses of the data from the short-term feeding model (two-way analysis of variance) revealed a significant relationship between mtDNA content and chronic ethanol feeding ($p < 0.001$) and mtDNA content and aging ($p < 0.001$). In addition, the data suggest that there is an underlying age-related mechanism of mtDNA depletion that can be advanced by the administration of agents, such as ethanol, that induce oxidative damage to mtDNA.

In conclusion ethanol feeding, both acute and chronic, depletes rat hepatic mtDNA via a mechanism that is independent of endonuclease G involvement. Moreover, data suggest that endonuclease G is not involved in mtDNA homeostasis. Furthermore, aging increases the susceptibility of hepatic mtDNA to depletion during short-term chronic ethanol feeding. The

consequences of these findings as they relate to the progression of alcoholic liver disease remain to be elucidated. In general, alcohol-induced liver injury in humans occurs only after at least 20 years of ethanol abuse, i.e., from approximately 40 years of age onward. In the rat, this would represent approximately 1 year of age, the same time point at which we observe marked depletions in mtDNA. The observations reported here indicate that it will be important to determine the effect of the age of the alcoholic on mtDNA levels, because it is likely that decreases in functional mtDNA will have a negative effect on mitochondrial energy conservation.

## CONCLUSION

The studies presented in this article clearly demonstrate the capacity of the mitochondrion to participate in generating oxidative stress in liver cells, particularly the hepatocyte, during ethanol oxidation. The increase in ROS production associated with ethanol metabolism damages mitochondrial components and also affects hepatocyte viability. Both acute and chronic ethanol exposure lead to increased mitochondrial production of ROS, with ROS stimulated by acute exposure being clearly related to generation of increased levels of NADH, which in turn favors superoxide production due to increased levels of the semiquinone forms of FMN and ubiquinone. Increased ROS production due to chronic consumption is most likely related to ethanol-elicited decreases in components of the electron transport chain that also give rise to higher steady-state levels of the semiquinone forms of FMN and ubiquinone.

It is quite possible that increased mitochondrial production of ROS in the presence of acute doses of ethanol actually causes much of the damage observed in the chronic alcohol consumer. Significant increases in protein oxidation are a consequence of ethanol exposure, as was emphasized in this symposium, and could contribute, at least in part, to the decrease in mitochondrial function that accompanies chronic ethanol consumption. Both protein oxidation and increases in mtDNA fragmentation can be considered consequences of increased ROS production associated with ethanol consumption. Although other ethanol metabolites, such as acetaldehyde, may contribute to mtDNA damage, the evidence presented in this symposium argues strongly for the participation of mitochondrial-generated ROS, which triggers the formation of oxidative products such as 8-hydroxydeoxyguanosine and initiates DNA strand breaks that disrupt the supercoiled structure of mtDNA. The products formed by oxidative damage to mtDNA evidently inhibit DNA polymerase γ, thus interfering with the mtDNA repair mechanism and further exacerbating oxidative damage to the mitochondrial genome. Ethanol-elicited damage to mtDNA is significantly greater in the older ethanol consumer, as has been demonstrated convincingly in the rodent. This suggests that the age of the alcohol abuser may be an etiological factor in the development of alcoholic liver disease, due to increased susceptibility of the mitochondrial genome to oxidative damage in older individuals. Indeed, in the alcoholic, large deletions of mtDNA similar to those seen in very old individuals are observed, suggesting that alcohol abuse leads to premature aging of the mitochondrial genome. This occurs in an environment of high oxidative stress, due to increased ROS production and compromised antioxidant mechanisms.

Ethanol-elicited oxidative stress, whether generated within or outside of the mitochondrion, also seems to contribute to hepatocellular apoptosis, which is also increased as a consequence of ethanol exposure. Previous studies suggest strongly that ROS production mediates ethanol-stimulated apoptosis in hepatocytes. The studies outlined in this article emphasize that ethanol-related apoptosis is mediated by mitochondria. Evidence was presented that acute ethanol exposure of hepatocytes, under conditions that stimulate mitochondrial ROS production, cause the association of Bax, a proapoptotic protein, with components of the PTP, concomitant with release of cytochrome *c* from mitochondria. These events are suppressed in the presence of antioxidants, and this provides further evidence that oxidative stress contributes not only to

oxidative damage within the cell, but also to an important and well established mechanism for hepatic cell death.

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